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(54) **COVALENT POLAR LIPID-PEPTIDE CONJUGATES FOR BIOLOGICAL TARGETING**
KOVALENTE POLARE LIPID-PEPTID-KONJUGATE FÜR BIOLOGISCHES TARGETING
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(56) References cited:
EP-A- 0 077 529
EP-A- 0 203 676
EP-A- 0 279 887
WO-A-89/10348
WO-A-90/00555
WO-A-90/10448
WO-A-91/01750
WO-A-91/16024
US-A- 4 780 455
US-A- 5 149 794

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Description

[0001] The present invention relates to polar lipid conjugates of antigenically-active peptides for facilitating the entry of peptides into cells and specific organelles within the cell for the specific production of immunological responses by such peptides. As such, this invention provides improved methods for vaccine production and *in vitro* vaccination against pathogenic microorganisms, and for alleviating autoimmune disease and ameliorating tissue and organ transplant rejection.

[0002] Vertebrate immunological responses to antigenic stimuli depend on a phenomenon called presentation of antigens. Presentation embodies the appearance of antigenic epitopes on the cell surface of antigen-presenting cells of the immune system in association with major histocompatibility complex (MHC) proteins. The MHC proteins are divided into 2 classes (I & II) whereby each class presents antigens derived from different sources. The two types of immune response corresponding to presentation *via* the two types of MHC molecules are said to be MHC class I or class II restricted. Both MHC class I and class II restricted immunity requires presentation of peptide antigens in association with MHC molecules (*see*, Abbas, Lichtman & Pober, 1991, *Cellular and Molecular Immunology* (W.B. Saunders Co.: Philadelphia), pp. 116-136 for review).

[0003] In the class I-restricted immune response, MHC class I molecules are associated with peptide antigens derived from proteins made intracellularly. Such proteins include proteins encoded by viruses and other intracellular pathogens. These proteins are degraded in the cytoplasm of infected cells, and the peptide products of this degradation transferred into the endoplasmic reticulum (ER) *via* the action of peptide-specific transporter molecules located in the ER membrane (*see*, Elliott *et al.*, 1990, Nature 348: 195-197; Parham, 1990, Nature 348: 674-675). Nascent MHC class I molecules synthesized in the ER are assembled into functional presenting proteins only in the presence of the appropriate peptide antigen. Fully assembled MHC class I complexes are then transported through the Golgi apparatus to the cell surface, where the antigen presenting complex can activate a cellular (T-cell mediated) immune response by interacting with $CD8^+$ cytotoxic T-cells (*see*, Falk *et al.*, 1990. Nature 348: 248-251; Falk *et al.*, 1991, Nature 351: 290-296).

[0004] Alternatively, in the MHC class II restricted immune response extracellular antigens (including free-living pathogens or protein components thereof) are engulfed by cells of the immune system (such as macrophages) by endocytosis, and transferred to the endosomal (lysosomal) compartment for degradation. Peptide products of such degradation may then associate with MHC class II molecules (which molecules lack the requirement of peptide association for cell-surface expression; *see*, Germain & Hendrix, 1991, Nature 353: 134-139) and appear on the cell surface (*see*, Sadegh-Nasseri & Germain, 1991, Nature 353: 167-170; Lanzavecchia *et al.*, 1992, Nature 357: 249-252). The MHC class II antigen-presenting pathway leads to the induction of a humoral (antibody-dependent) immune response and the activation of $CD4^+$ T-helper cells.

[0005] In the preparation and use of vaccines to provoke immunity to pathogenic organisms, the appropriate MHC restriction is achieved using alternative strategies (*see*, Abbas, Lichtman & Pober, 1991, *Cellular and Molecular Immunology* (W.B. Saunders Co.: Philadelphia), p. 315 *et seq.* for review). MHC class I restricted immunity requires the use of attenuated pathogenic organisms (usually viruses) which non-productively infect host cells. The protein antigens of the pathogenic organism is degraded intracellularly, and the peptide antigens produced transduced, to the ER and assembled into functional MHC class I presentation complexes. Presentation of antigen to cytotoxic T-cells results an cellular immunity against the pathogenic microorganism.

[0006] Vaccination using the MHC class II-restricted route involves inoculation with inactivated (*e.g.*, chemically inactivated) pathogens, which are then engulfed by macrophages and lysosomally degraded intracellularly. Peptide antigens associated with the appropriate MHC class II complex are then presented to T-helper cells, which cells release cytokines and other immune system stimulating factors, which activate antibody-producing B cells specific for the peptide antigen presented.

[0007] Both routes to producing immunity to pathogenic organisms require degradation and selection of the appropriate peptide antigen by the cells of an animals immune system *in vivo*. This allows for variability in the efficacy of production of the immune response, and in the case of the use of attenuated viruses, the possibility for reversion to pathogenicity (with the result that the vaccine causes the disease it was meant to forestall). There is a need, therefore, for developing methods to efficiently deliver peptide antigens directly to cells of the immune system for presentation to T-cells in association with the appropriately restricted MHC complex.

[0008] Similarly, autoimmune disease is related to the presentation and immunological recognition of peptide antigens derived from endogenous cellular proteins (called self-antigens; *see*, Jardetzky *et al.*, 1991, Nature 353: 326-329; Faustman *et al.*, 1991, Science 254: 1756-1771). Self-antigens may be presented by either the MHC class I or class II restricted pathway, resulting in either cellular or humoral autoimmunity, or both. There is a need to develop methods and reagents to block presentation of self-antigens, thereby ameliorating or preventing the onset of progression of autoimmune disease.

[0009] In addition, tissue or organ transplant rejection is mediated by both MHC class I and class II restricted

immune response. Non-self antigens are processed and recognized by the immune system of the transplant recipient, causing an immunological attack on the transplant resulting in its rejection by the host. Current methodologies of inhibiting transplant rejection involve suppressing the immune system indiscriminately with drugs such as cyclosporin A. These methods leave the host immune-compromised and at risk for adventitious infection by pathogens. There is a need for methods of selectively blocking host MHC restricted immune response against tissue and organ transplants which do not result in general immune suppression.

[0010] The use of peptide antigens as immunogens has been attempted in the prior art, with limited success *in vivo*.

[0011] Hopp, 1984, Mol. Immunol. 21: 13-16 disclosed the use of a synthetic hepatitis viral antigen acylated with a fatty acid moiety as an *in vivo* immunogen.

[0012] Neurath *et al.*, 1984, J. Gen. Virol. 65: 1009-1014 utilize hepatitis surface antigen-derived peptide immunogens that are chemically fixed to liposomes *in vitro*.

[0013] Deres *et al.*, 1989, Nature 342: 561-564 disclose the use of influenza peptide epitopes chemically linked to lipoprotein adjuvants as immunogens *in vivo*.

[0014] Seifert *et al.*, 1990, J. Biochem. 267: 795-802 teach the use of lipoprotein-derived synthetic antigens for activating human neutrophils *in vitro*.

[0015] Brynestad *et al.*, 1990, J. Virol. 64: 680-685 use palmitoylated peptide antigens derived from herpes simplex virus glycoprotein D as immunogens *in vivo*.

[0016] Wiesmüller *et al.*, 1991. Immunology 72: 109-113 demonstrate that synthetic lipoprotein analogues stimulate cytokine release and activate B cells and macrophages *in vitro*.

[0017] Frisch *et al.*, 1991, Eur. J. Immunol. 21: 185-193 describe the use of a histone H3 derived, hexapeptide antigen covalently attached to phosphatidylethanolamine and encapsulated into liposomes for immunizing mice *in vivo*.

[0018] Peptides have also been used for blocking an immune response.

[0019] Vandenbark *et al.*, 1989, Nature 341: 841-844 demonstrate that a peptide derived from the T-cell receptor Vβ8 chain can be used to block experimentally-induced autoimmune encephalitis (EAE) *in vivo*.

[0020] Lamont *et al.*, 1990, J. Immunol. 144: 2493-2498 disclose peptide MHC class II inhibitors of antigen presentation.

[0021] Guéry *et al.*, 1992, J. Exp. Med. 175: 1345-1352 demonstrate inhibition of antigen presentation *in vivo* using a blocking peptide antigen.

[0022] DeMagriatis *et al.*, 1992, Cell 68: 625-634 show the use of an influenza peptide to block T-cell mediated immunity *in vitro*.

[0023] EP 0 077 529 (Speiser) discloses conjugates of drugs and glycerolipids for administration in the form of liposomes. One of the drugs suggested be made as a conjugate is insulin.

[0024] WO90/00555 (Vical Inc) teaches conjugation of antiviral nucleosides and lipids in order to enhance delivery of the nucleosides to virally, particularly HIV infected cells. Specificity in cell uptake of the conjugates is said to be achieved using liposomes because of the preferential uptake of liposomes exhibited by the macrophages which usually harbour HIV.

[0025] WO90/10448 (Genentech, Inc) concerns antisense oligonucleotides linked to lipids and how to achieve cellular uptake of these conjugates. In order to achieve cell specific intracellular location of free oligonucleotides particular lipid-oligonucleotide linkages are proposed.

[0026] Frisch B *et al* (1991) Eur. J. Immunol 21: 185-193 reports on how the immunogenicity of small peptides can be enhanced by linking them covalently to the surface of pre-formed liposomes.

[0027] In first aspect the present invention provides a conjugate of an immunologically active peptide, covalently linked to a polar lipid carrier molecule, in other than liposome form, excluding a conjugate of insulin.

[0028] The polar lipid is preferably selected from the group consisting of sphingosine, ceramide, phosphatidylcholine, phosphatidyl glycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, cardiolipin and phosphatidic acid, sphingomyelin and other sphingolipids.

[0029] The peptide may be a peptide fragment of pathogenic and microbially-derived proteins. When such a conjugate is in use as a pharmaceutical, the peptide becomes presented via the major histocompatibility complex class I antigen presentation pathway. Alternatively, when such a conjugate is in use as a pharmaceutical, the peptide becomes presented via the major histocompatibility complex class II antigen presentation pathway.

[0030] In one preferred embodiment the peptide is a self-antigen blocking peptide. In another preferred embodiment the peptide is a nonself-antigen blocking peptide.

[0031] The antigenically-active peptide may comprise 4 to 100 amino acids including amino acid analogues and derivatives thereof.

[0032] In other embodiments, the peptide may be covalently linked via a spacer moiety, said spacer comprising a functional linker group at each end thereof. One functional linker group may be weak and the other functional linker group strong.

[0033] The spacer may allows for the facilitated hydrolytic release or facilitated enzymatic release of the peptide, at an intracellular site. A preferred spacer moiety is a peptide of formula $(\text{amino acid})_n$, where n is in the range 2 to 25 and the peptide is a polymer of a particular amino acid.

[0034] In second aspect the invention provides a pharmaceutical composition comprising a conjugate as hereinbefore defined and a pharmaceutically acceptable carrier.

[0035] In third aspect the invention provides a vaccine comprising a conjugate as hereinbefore defined and a pharmaceutically acceptable carrier.

[0036] In fourth aspect of the invention provides the use of a conjugate as hereinbefore defined for the manufacture of a vaccine for the prevention of infection by a pathogenic organism, eg virus, bacterium, fungus or protozoan.

[0037] In fifth aspect, when the peptide is a self-antigen blocking peptide, the invention provides the use of a conjugate as hereinbefore defined for the manufacture of a medicament for prevention of autoimmune disease.

[0038] In sixth aspect, when the peptide is a nonself-antigen blocking peptide, the invention provides the use of a conjugate as hereinbefore defined for the manufacture of a medicament for preventing tissue or organ transplant rejection.

[0039] In seventh aspect the invention provides the use of a conjugate of an immunologically active peptide, covalently linked to a polar lipid carrier molecule and in other than liposome form, for the manufacture of a composition for the facilitated entry of the peptide into a selected cell or sub-cellular organelle. Preferred embodiments of this aspect of the invention are as hereinbefore defined.

[0040] The present invention is directed to methods for eliciting or inhibiting an immune response in an animal, preferably a human. The invention provides reagents comprising antigenically-active peptides covalently linked to polar lipid carrier molecules. Conjugation of the antigenically-active peptides to the polar lipid can be mediated by a spacer moiety. The choice of polar lipid conjugated to the antigenically-active peptides of the invention will influence the intracellular size to which such peptide/lipid conjugates are targeted. Methods for using the reagents of the invention are also provided.

[0041] This invention has the specific advantage of facilitating the entry of antigenically-active peptides into cells of the immune system *via* a polar lipid carrier, allowing introduction of such peptides into cells in the absence of intracellular production of the peptides and without requiring endocytosis of such peptides into the degradative compartment of such cells. As disclosed herein, the invention comprehends a polar lipid/peptide conjugate wherein the polar lipid will selectively associate with certain biological membranes, and facilitate intracellular localization of the peptides therein.

[0042] The polar lipid may also be conjugated to the antigenically-active peptide through use of a spacer, which may act to release the peptide from the lipid, target the conjugate to the proper intracellular compartment, or perform other functions to maximize the effectiveness of immunological processing by the cell.

[0043] This type of conjugate is advantageous for a number of reasons. First, the ability of this invention to allow the entry of antigenically-active peptides into cells at pharmokinetic rates eliminates the requirement using traditional vaccination methods for intracellular synthesis of viral peptide antigens destined for presentation *via* the major histocompatibility complex class I antigen presentation pathway. Second, for antigens presented *via* the major histocompatibility complex class II antigen presentation pathway, the specific antigenic epitope can be delivered to the lysosomal compartment of the cell for association with nascent MHC class II molecules without the need for intracellular proteolysis of the cognate protein of the peptide antigen. Third, the reagents of the invention may incorporate a spacer region that can be varied and thereby allow an immunologically-relevant rate of antigen release in antigen-presenting cells.

[0044] The invention may provide a composition of matter comprising a peptide, a polar lipid carrier, two linker functional groups and a spacer, wherein the spacer has a first end and a second end and wherein the polar lipid is attached to the first end of the spacer through a first linker functional group and the peptide is attached to the second end of the spacer through a second linker functional group. In a preferred embodiment, the peptide is an antigenically active peptide. In another preferred embodiment, the spacer allows the peptide to act without being released at an intracellular site. In this embodiment of the invention, the covalent attachment of the first linker functional group to the first end of the spacer is weak and the covalent attachment of the second linker functional group to the second end of the spacer is strong; alternatively, both covalent attachments are strong. In other embodiments, the spacer facilitates hydrolytic or enzymatic release of the peptide at an intracellular site. In this embodiment, the covalent attachment of the first linker functional group to the first end of the spacer is strong and the second linker functional group attached to the second end of the spacer is weak. Preferred polar lipids include sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin and phosphatidic acid.

[0045] A second embodiment of this aspect of the invention provides a composition of matter comprising a peptide having a first functional linker group, and a polar lipid carrier having a second functional linker group, wherein the peptide is covalently linked to the polar lipid carrier by a chemical bond between the first and second functional linker groups. In a preferred embodiment, the peptide is an antigenically active peptide. Preferred first and second functional linker groups each independently include a hydroxyl group, a primary or secondary amino group, a phosphate group or substituted derivatives thereof and a carboxylic acid group. Preferred polar lipids include sphingosine, ceramide, phos-

phatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin and phosphatidic acid.

[0046] In another embodiment of this aspect of the invention, the spacer molecule is a peptide of formula (amino acid), wherein n is an integer between 2 and 25, preferably wherein the peptide comprises a polymer of a particular amino acid.

[0047] The invention provides a method of immunizing an animal against a pathogenic microorganism, comprising the step of inoculating the animal with a reagent that is a composition of matter of the invention in a pharmaceutically acceptable carrier in an amount sufficient to elicit an immunological response in the animal. The preferred animal is a human being.

[0048] The invention also provides a method of alleviating autoimmune disease in an animal, comprising the step of inoculating the animal with a reagent that is a composition of matter of the invention in a pharmaceutically acceptable carrier in an amount sufficient to inhibit the autoimmune response in the animal. The preferred animal is a human being.

[0049] The invention further provides a method for preventing tissue or organ transplant rejection in an animal, comprising the step of inoculating the animal with a reagent that is a composition of matter of the invention in a pharmaceutically acceptable carrier in an amount sufficient to inhibit transplant rejection in the animal. The preferred animal is a human being.

[0050] Preferred embodiments of the invention will now be described in detail with reference to the accompanying drawings in which:

Figure 1 depicts the synthetic scheme put forth in Example 1.
Figure 2 depicts the synthetic scheme put forth in Example 2.
Figure 3 depicts the synthetic scheme put forth in Example 3.
Figure 4 depicts the synthetic scheme put forth in Example 4.
Figure 5 depicts the synthetic scheme put forth in Example 5.
Figure 6 depicts the synthetic scheme put forth in Example 6.
Figure 7 depicts the synthetic scheme put forth in Example 7.

[0051] The present invention provides methods and compositions of matter for facilitating the entry of antigenically-active peptides into cells and for delivering such peptides to the appropriate intracellular organelles for immunological processing and antigen presentation. This is achieved by conjugating the desired antigenically-active peptide to a polar lipid carrier and administering this conjugate to an animal by standard techniques. The antigenically-active peptides may comprise peptide hormones and peptide fragments of proteins, particularly pathogenic and microbially-derived proteins, useful, for example, as vaccines and otherwise.

[0052] The compositions of matter provided by the invention comprise the immunologically active peptides of the invention covalently linked to a polar lipid carrier. A polar lipid carrier, as defined herein is intended to mean any polar lipid having an affinity for, or capable of crossing, a biological membrane, including but not limited to sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin, phosphatidic acid, sphingomyelin and other sphingolipids, as these terms are understood in the art (*see*, Lehninger, *Biochemistry*, 2d ed., Chapters 11 & 24, Worth Publishers: New York, 1975).

[0053] The compositions of matter of the invention may be further comprised of a spacer moiety comprising a first end and a second end, each end of the spacer having a functional linking group. For the purposes of this invention, the term "spacer" or "spacer moiety" is intended to encompass any chemical entity that links the peptide and the polar lipid. Such spacer moieties may be designed to facilitate the attachment of the conjugates of the invention to a target cell, or to facilitate, influence, modulate or regulate the release of the peptide at the desired target site. Such spacers may also facilitate enzymatic release at certain intracellular sites. Spacer groups, as described herein, include, but ate not limited to aminohexanoic acid, polyglycine, polyamides, polyethylenes, and short functionalized polymers having a carbon backbone which is from one to about twelve carbon molecules in length. Particularly preferred embodiments of such spacer moieties comprise peptides of formula $(\text{amino acid})_n$, wherein n is an integer between 2 and 25 and the peptide is a polymer of a particular amino acid.

[0054] The term "linker functional group" is defined herein as any functional group for covalently binding the polar lipid carrier or peptide to the spacer group. These groups can be designated either "weak" or "strong" based on the stability of the covalent bond which the linker functional group will form between the spacer and either the polar lipid carrier or the peptide. The weak functionalities include, but are not limited to phosphoramide, phosphoester, carbonate, amide, carboxyl-phosphoryl anhydride, ester and thioester. The strong functionalities include, but are not limited to ether, thioether, amine, amide and ester. The use of a strong linker functional group between the spacer group and the peptide will tend to decrease the rate at which the peptide will be released at the target site, whereas the use of a weak linker functional group between the spacer group and the peptide may act to facilitate release of the peptide at the target site. Enzymatic release is, of course, also possible, but such enzyme-mediated modes of release will not necessarily be cor-

related with bond strength in such embodiments of the invention. Spacer moieties comprising enzyme active site recognition groups, such as spacer groups comprising peptides having proteolytic cleavage sires therein, are envisioned as being within the scope of the present invention.

[0055] Experimentally, it was found that fluorescent ceramide is distributed differentially in different cells. These results suggest that by the proper choice of polar lipid conjugate, intracellular targeting of antigenically-active peptides can be achieved. This result enables the delivery of antigenically-active peptides to nascent major histocompatibility complex protein molecules of both types (class I and class II). Thus, antigen presentation of such antigenically-active peptides can be achieved, allowing activation of both humoral and cellular immunity.

[0056] The invention specifically provides methods for preparing and administering vaccines against pathological microorganisms, and compositions comprising such vaccines. Vaccines provided by the invention include but are not limited to vaccines against poliovirus, measles virus, rabies virus, the rubella virus, human immunodeficiency virus, Epstein-Barr virus, varicella zoster, herpes simplex virus, hepatitis virus, human papilloma virus, and the microorganisms responsible for diphtheria, malaria, scarlet fever, viral and bacterial pneumonia, whooping cough, scrapie, and other diseases.

[0057] Alternatively, pathological conditions (such as autoimmune disease) may be alleviated by the selective blocking of self-antigen presentation by the administration of the appropriate blocking peptides covalently linked to an appropriate polar lipid carrier. Autoimmune diseases intended for this treatment include but are not limited to diabetes type I, lupus erythematosus, rheumatoid arthritis, encephalomyelitis, Hashimoto's disease, oophoritis, orchiditis, myasthenia gravis, polyneuritis, polymyositis, dermatomyositis, scleroderma, rheumatoid carditis, Sjögen's syndrome, and autoimmune hemolytic anemias.

[0058] Similarly, tissue and organ transplantation rejection can be inhibited by the selective blocking of nonself-antigen presentation by the administration of the appropriate blocking peptides covalently linked to an appropriate polar lipid carrier. The methods of this invention are intended to be useful in inhibiting rejection of transplanted organs and tissues including kidney, liver, pancreas, lung, heart, cornea, bone marrow, skin, endocrine organs, and portions of the gastrointestinal tract, although this is not intended to be an exhaustive listing of all the uses for this aspect of the invention.

[0059] Animals to be treated with polar lipid-antigenically active peptide conjugates using the methods of the invention are intended to include all vertebrate animals, preferrably domesticated animals, such as cattle, horses, goats, sheep, fowl, fish, household pets, and others, as well as wild animals, and most preferably humans.

[0060] An antigenically-active peptide, as used herein, is defined as including, but not necessarily limited to any peptide, comprising 4-100 amino acids (including naturally-occurring amino acids, amino acid analogues and derivatives thereof), that is capable of eliciting or inhibiting and immunological response in an animal, preferably a human being. More specifically, such antigenically-active peptides are characterized by their capacity to induce, augment or block (i.e., down-regulate) humoral and/or cellular immune responses *in vivo*. Such peptides include peptides whose amino acid sequence is known and can be chemically synthesized *in vitro*, or produced using recombinant genetic means, as well as mixtures of such peptides. Alternatively, antigenic proteins can be chemically or enzymatically degraded *in vitro*, and mixtures of peptides so produced then used for preparing peptide-polar lipid conjugates of the invention. Covalently-linked multimers of such antigenically-active peptides are also encompassed by the invention.

[0061] Representative antigenically-active peptide sequences include but are not limited to:

Vaccines	
PKYVKQNTLKLAT	(influenza virus hemagglutinin, residues 307-319)
IYATVAGSL	(influenza virus hemagglutinin, residues 523-531)
QYIKANSKFIGITE	(tetanus toxoid, residues 830-843)
SLSDLRGYVYQGLKSGNVS	(VSV nucleocapsid, residues 47-65)
TYQRTRALVRTG	(influenza virus nucleoprotein, residues 147-158)
IASNENMETMESSTLE	(influenza virus nucleoprotein, residues 365-380)
SRYWAIRTR	(influenza virus nucleoprotein, residues 383-391)
SYVPSAEQI	(*P. yoelii* CSP, residues 276-288)
SYIPSAEKI	(*P. berghi* CSP, residues 249-260)
NANP	(*P. falciparum* CSP)
ILKEPVHGV	(HIV reverse transcriptase, residues 461-469)
FLQSRPEPT	(HIV *gag* protein, residues 446-454)
AMQMLKE	(HIV *gag* protein, residues 193-199)
PIAPGQMRE	(HIV *gag* protein, residues 219-227)
QMKDCTERQ	(HIV *gag* protein, residues 418-426)
KRWIILGLNKIV	(HIV *gag* protein, residues 265-276)
GRAFVTIGK	(HIV gp120, residues 314-322)
CCTKPTEGNCTC	(hepatatis B surface antigen, residue 138-149)
KYALAEASLKMAEPNQFRGKELP	(HSV glycoprotein D-1, residues 1-23)
KYALAEPSLKMAEPNQFRGKNLP	(HSV glycoprotein D-2, residues 1-23)
RYNRNAVPNLRGELQVLAQKVARTLP	(FMDV VP1, residues 135-160)
SGVENPGGYCL	(lymphocyte choriomeningitis virus glycoprotein, residues 272-282)

Autoimmunity	
DMGHGLRLIHYSYDVNSTEKG	(T-cell receptor Vβ8)
APGGTLQQLFYSFNVGQSELV	(T-cell receptor Vβ8)
GRTQDENPVVHPPKNIVTPRTPPP	(myelin basic protein)
ASQKRPSQRHG	(myelin basic protein)
IRGERA	(human histone H3)
RRYQKSTEL	(human histone H3)
RRIKEIVKK	(human heat shock protein 89α)
RRVKEVVKK	(human heast shock protein 89β)
NLLDGDPRDFVDNS	(EGF receptor, residues 516-529)
PEFLEQRRAAVDTYC	(Es β chain)
Transplantation	
RYLENGKET	(HLA-A24, residues 170-179)
RYLKNGKET	(HLA-Cw3, residues 170-179)
PPKTHVTHHP	(HLA-B27, residues 182-191)
GSHSMRYFHTSV	(HLA-B27, residues 1-12)
SYFPEITHI	(self peptide[1])
KRFEGLTQR	(self peptide[2])
RRFTRPEH	(self peptide[2])
RRISGVDRY	(self peptide[2])
ARLFGIRAK	(self peptide[2])

([1] Falk *et al.*, 1991, Nature 351: 290-296)
([2] Jardetzky *et al.*, 1991, Nature 353: 326-329)

[Single letter abbreviations for amino acids can be found in Zubay, 1988, Biochemistry 2d ed., (MacMillan Publishing: New York), p. 33.]

[0062] The following Examples illustrate certain aspects of the above-described method and advantageous results. The following examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1

[0063] An antigenically-active peptide is conjugated to sphingosine as follows. Sphingosine is reacted with 1,3 *bis*(trimethylsilyl)urea as described by Verbloom *et al.* (1981, Synthesis 1032: 807-809) to give a trimethylsilyl derivative of sphingosine. The sphingosine derivative is then conjugated with the antigenically-active peptide in which the terminal amine and any of the constituent amino acid sidechain reactive amines are covered by *tert*-butoxycarbonyl (*t*-Boc) protecting groups in the presence of diethylazodicarboxylate (DEAD) and triphenyl phosphine as described by Kishimoto (1975, Chem. Phys. Lipids 15: 33-36). The sphingosine/peptide conjugate is then reacted in the presence of pyridine hydrofluoride as described by Matsuura *et al.* (1976, J. Chem. Soc. Chem. Comm. pg. 451-459) to remove the *t*-Boc protecting group, to yield the antigenically-active peptide covalently linked to sphingosine through an amide bond. This reaction scheme is illustrated in Figure 1.

EXAMPLE 2

[0064] An antigenically-active peptide compound is conjugated to ceramide *via* an oligomeric 3-hydroxy-propanoic acid spacer wherein a first end of the oligomeric spacer is conjugated to ceramide through an ester functional group, and wherein the antigenically-active peptide is conjugated to a second end of the polyester spacer through an amide linkage to the amino terminus of the antigenically-active peptide. The polyester spacer is first obtained, having a car-

boxyl group at a first end and a triphenylmethyl group esterified to a second end. This spacer is conjugated to ceramide at its first end through an ester functional linker group according to the method of Anderson *et al.* (1963, J. Am. Chem. Soc. 85: 3039). This compound is then conjugated through the second end of the spacer compound to the antigenically-active peptide by means of a amide linkage according to the method of Verbloom *et al.* (1981, Synthesis 1032: 807-809). This reaction scheme is illustrated in Figure 2.

EXAMPLE 3

[0065] An antigenically-active peptide compound wherein phosphatidic acid, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol or phosphatidylethanolamine is linked through a phosphoester linker functional group to the antigenically-active peptide. Phosphatidic acid, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol or phosphatidyl ethanolamine is conjugated to the carboxyl terminus of the antigenically-active peptide according to the method of Salord *et al.* (1986, Biochim. Biophys. Acta 886: 64-75). This reaction scheme is illustrated in Figure 3.

EXAMPLE 4

[0066] An antigenically-active peptide compound is prepared wherein aminohexanoyl sphingosine is conjugated to the carboxyl terminus of peptide the antigenically-active peptide. Aminohexanoyl sphingosine is conjugated with the antigenically-active peptide according to the method of Kishimoto (1975, Chem. Phys. Lipid 15: 33-36). This reaction scheme is illustrated in Figure 4.

EXAMPLE 5

[0067] An antigenically-active compound is prepared consisting of ceramide conjugated to a first end of an antigenically-active peptide through an ester linker functional group or an amide linker functional group. The antigenically-active peptide has both a carboxyl terminus and an amino terminus, the amino terminus being protected by a *t*-Boc group. The antigenically-active peptide is conjugated through its carboxyl terminus to ceramide forming an ester linkage, as described by Anderson *et al.* (1963, J. Chem. Soc. Chem. Comm. 85: 3039). The amino terminus of the antigenically-active peptide is then deprotected according to the method of Matsuura *et al.* (1976, J. Chem. Soc. Chem. Comm. pg. 451). This reaction scheme is illustrated in Figure 5.

EXAMPLE 6

[0068] An antigenically-active peptide is prepared wherein sphingosine is conjugated to the amino terminus of the antigenically-active peptide through an adipic acid spacer. The primary amino and hydroxyl groups of sphinogosine are acylated by reaction with adipic acid monomethyl ester overnight at 40-50°C, followed by base hydrolysis of the ester (in 0.1N methanolic KOH). The free hydroxyl group of this intermediate is protected using *t*-butyldimethylsilane (TBDMS) by reaction overnight at room temperature. The antigenically-active peptide is then covalently linked to the free carboxyl end of the adipic acid spacer activated by reaction overnight at 40-50°C in the presence of carbonyl diimidazole (the details of this reaction may be found in *Enzyme*, vol.18, p. 310. 1974). The TBDMS protecting groups are then removed using terrabutylammoniumfluoride to yield the antigenically-active peptide product. This reaction scheme is illustrated in Figure 6.

EXAMPLE 7

[0069] An antigenically-active peptide compound is prepared wherein phosphatidic acid, phosphatidyl choline, phosphatidyl glycerol or phosphatidyl ethanolamine is linked through die *sn*-2 or *sn*-1 hydroxyl of the lysophospholipid to the antigenically-active peptide using the methods of Martin & Josey (1988, Tetrahedron Lett. 29: 3631-3634). This reaction scheme is illustrated in Figure 7. Briefly, the antigenically active peptide (whether or not covalently linked to a spacer moiety), or alternatively a fatty acid, is conjugated to the *sn*-1 hydroxyl of 3-*sn*-benzyl glycerol by reaction at 0°C in the presence of dimethylaminopyridine/ dicyclohexylcaxbodiimide/ methylene chloride (DMAP/DCC/CH_2Cl_2). A fatty acid, or alternatively the antigenically-active peptide (whether or not covalently linked to a spacer moiety), is then conjugated to the *sn*-2 hydroxyl of 3-*sn*- benzyl-1-*sn*-substituted glycerol at 20°C in the presence of DMAP/DCC/CH_2Cl_2. The 3-*sn* position is then deprotected at 45°C in ethanol/acetic acid in the presence of platinum black and H_2. The appropriate polar head group is then phospho-esterified to the *sn*-3 position in the presence of phonyldichlorophosphite/ diisopropylethylamino/ tetrahydrofuran at -78°C.

[0070] Alternatively, the antigenically-active peptide (whether or not covalently linked to a spacer moiety), can be

conjugated to phospholipid following enzymatic deacylation of a diacylphospholipid with phospholipase A_2 using the method of Eibi *et al.* (1983, Meth. Enzymol. 98: 623).

EXAMPLE 8

[0071] Antigenically-active peptide-polar lipid conjugates of the invention are used as follows. For use as a vaccine, the conjugate, the naked peptide and a negative control (saline) are administered to an animal using both optimal and suboptimal dosages and the most appropriate route of administration. After an optimal time period (determined from the nature of the immunological response to be elicited), both sera and lymphoid cells are collected from the animal and tested for reactivity to the antigen. Lymphoid cells are isolated using conventional methods (Ficoll-Hypaque density gradient centrifugation) and tested for cytotoxic activity against control autologous macrophage/monocyte preparations exposed to and subsequently presenting the original peptide antigen. Testing is performed using the ^{51}Cr release assay of Malkovsky *et al.* (1982, Nature 300: 652-655). Antibody response is tested using standard radioimmunoassay methods (*see*, Brenner *et al.*, 1984, Eur. J. Immunol. 14: 1021-1027). Briefly, Linbro flexible plates are coated with the specific peptide antigen by overnight incubation of a peptide solution (1 mg/mL) in phosphate buffered saline (PBS). Nonspecific binding is then blocked by treatment of the plates with a solution of 0.2% bovine serum albumin and 0.2% gelatin in PBS. The plates are then washed, sera to be tested is then added, and the plates rewashed after incubation of the sera on the plates. ^{125}I-labeled anti-IgG and anti-IgM antibodies are then added, the plates washed and bound radioactivity counted. The amount of peptide-antigen specific antibody present on each plate is then calculated relative to a standard curve prepared with known amounts of anti-peptide antibody. From these experiments the titre of specific antibody against each peptide antigen is calculated for each experimental sera tested.

[0072] For use in preventing transplant rejection or to treat autoimmune disease, the appropriate administration protocol is determined by vaccination of an animal as described above. After an empirically-determined optimal time period (determined from the nature of the immunological response to be elicited), both sera and lymphoid cells are collected from the animal and tested for reactivity to either self-antigen (for autoimmune disease uses) or heterologous transplantation antigens, as described above.

[0073] It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

Claims

1. A conjugate of an immunologically active peptide, covalently linked to a polar lipid carrier molecule, in other than liposome form, excluding a conjugate of insulin.

2. A conjugate as claimed in claim 1, wherein the polar lipid is selected from the group consisting of sphingosine, ceramide, phosphatidylcholine, phosphatidyl glycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, cardiolipin and phosphatidic acid, sphingomyelin and other sphingolipids.

3. A conjugate as claimed in claim 1 or claim 2, wherein the peptide is a peptide fragment of pathogenic and microbially-derived proteins.

4. A conjugate as claimed in any of claims 1 to 3, wherein in use as a pharmaceutical the peptide becomes presented via the major histocompatibility complex class I antigen presentation pathway.

5. A conjugate as claimed in any of claims 1 to 3, wherein in use as a pharmaceutical the peptide becomes presented via the major histocompatibility complex class II antigen presentation pathway.

6. A conjugate as claimed in claim 1 or claim 2, wherein the peptide is a self-antigen blocking peptide.

7. A conjugate as claimed in claim 1 or claim 2, wherein the peptide is a nonself-antigen blocking peptide.

8. A conjugate as claimed in any preceding claim, wherein the antigenically-active peptide comprises 4 to 100 amino acids including amino acid analogues and derivatives thereof.

9. A conjugate as claimed in any preceding claim, wherein the peptide is covalently linked via a spacer moiety, said spacer comprising a functional linker group at each end thereof.

10. A conjugate as claimed in claim 9, wherein one functional linker group is weak and the other functional linker group is strong.

11. A conjugate as claimed in claim 9 or claim 10, wherein the spacer allows the facilitated hydrolytic release or facilitated enzymatic release of the peptide, at an intracellular site.

12. A conjugate as claimed in any of claims 9 to 11, wherein the spacer moiety is a peptide of formula $(\text{amino acid})_n$, where n is in the range 2 to 25 and the peptide is a polymer of a particular amino acid.

13. A pharmaceutical composition comprising a conjugate as defined in any of claims 1 to 12 and a pharmaceutically acceptable carrier.

14. A vaccine comprising a conjugate as defined in any one of claims 1 to 12 and a pharmaceutically acceptable carrier.

15. The use of a conjugate as defined in claims 3 to 5 or 8 for the manufacture of a vaccine for the prevention of infection by a pathogenic organism, eg virus, bacterium, fungus or protozoan.

16. The use of a conjugate as defined in claim 6 for the manufacture of a medicament for prevention of autoimmune disease.

17. The use of a conjugate as defined in claim 7 for the manufacture of a medicament for preventing tissue or organ transplant rejection.

18. The use of a conjugate of an immunologically active peptide, covalently linked to a polar lipid carrier molecule and in other than liposome form, for the manufacture of a composition for the facilitated entry of the peptide into a selected cell or sub-cellular organelle.

19. The use as claimed in claim 18, wherein the conjugate is as further defined in any of claims 2 to 12.

Patentansprüche

1. Konjugat eines immunologisch aktiven Peptids, das kovalent mit einem polaren Lipid-Trägermolekül in einer Nicht-Liposomen-Form verknüpft ist, wobei ein Insulin-Konjugat ausgenommen ist.

2. Konjugat gemäß Anspruch 1, wobei das polare Lipid ausgewählt ist aus der Gruppe bestehend aus Sphingosin, Ceramid, Phosphatidylcholin, Phosphatidylglycerin, Phosphatidylethanolamin, Phosphatidylinositol, Phosphatidylserin, Cardiolipin, Phosphatidsäure, Sphingomyelin und anderen Sphingolipiden.

3. Konjugat gemäß Anspruch 1 oder 2, wobei das Peptid ein Peptidfragment pathogener und mikrobiell abgeleiteter Proteine ist.

4. Konjugat gemäß einem der Ansprüche 1 bis 3, wobei das Peptid bei einer Verwendung als pharmazeutischer Wirkstoff über den Präsentationsweg des Haupt-Histokompatibilitätskomplex-Klasse I-Antigens präsentiert wird.

5. Konjugat gemäß einem der Ansprüche 1 bis 3, wobei das Peptid bei einer Verwendung als pharmazeutischer Wirkstoff über den Präsentationsweg des Haupt-Histokompatibilitätskomplex-Klasse II-Antigens präsentiert wird.

6. Konjugat gemäß Anspruch 1 oder 2, wobei das Peptid ein Eigenantigen-blockierendes Peptid ist.

7. Konjugat gemäß Anspruch 1 oder 2, wobei das Peptid ein Nicht-Eigenantigen-blockierendes Peptid ist.

8. Konjugat gemäß einem der vorherigen Ansprüche, wobei das als Antigen aktive Peptid 4 bis 100 Aminosäuren umfasst, einschließlich Aminosäure-Analoga und -derivate davon.

9. Konjugat gemäß einem der vorherigen Ansprüche, wobei das Peptid kovalent über einen Distanzstück-Rest verknüpft ist und das Distanzstück eine funktionelle Verbindungsgruppe an jedem Ende davon umfasst.

10. Konjugat gemäß Anspruch 9, wobei eine funktionelle Verbindungsgruppe schwach und die andere funktionelle Verbindungsgruppe stark ist.

11. Konjugat gemäß Anspruch 9 oder 10, wobei das Distanzstück die erleichterte hydrolytische Freisetzung oder erleichterte enzymatische Freisetzung des Peptids an einer intrazellulären Stelle erlaubt.

12. Konjugat gemäß einem der Ansprüche 9 bis 11, wobei der Distanzstück-Rest ein Peptid der Formel $(Aminosäure)_n$ ist, in der n einen Wert im Bereich von 2 bis 25 darstellt, und das Peptid ein Polymer einer bestimmten Aminosäure ist.

13. Pharmazeutische Zusammensetzung, umfassend das Konjugat gemäß einem der Ansprüche 1 bis 12 und einen pharmazeutisch verträglichen Träger.

14. Vaccine, umfassend das Konjugat gemäß einem der Ansprüche 1 bis 12 und einen pharmazeutisch verträglichen Träger.

15. Verwendung des Konjugats gemäß den Ansprüchen 3 bis 5 oder 8 für die Herstellung eines Impfstoffs zur Prävention einer Infektion durch einen pathogenen Organismus, wie ein Virus, ein Bakterium, ein Pilz oder ein Protozoon.

16. Verwendung des Konjugats gemäß Anspruch 6 für die Herstellung eines Medikaments zur Prävention einer Autoimmunerkrankung.

17. Verwendung des Konjugats gemäß Anspruch 7 für die Herstellung eines Medikaments zur Prävention der Abstoßung eines Gewebe- oder Organtransplantats.

18. Verwendung eines Konjugats eines immunologisch aktiven Peptids, das kovalent in einer Nicht-Liposomen-Form mit einem polaren Lipid-Trägermolekül verknüpft ist, für die Herstellung einer Zusammensetzung zum erleichterten Eintritt des Peptids in eine ausgewählte Zelle oder ein sub-zelluläres Organell.

19. Verwendung gemäß Anspruch 18, wobei das Konjugat ferner in einem der Ansprüche 2 bis 12 definiert ist.

Revendications

1. Conjugué d'un peptide immunologiquement actif, lié de manière covalente à une molécule transporteuse lipidique polaire, sous une forme différente d'une forme liposomée, excluant un conjugué d'insuline.

2. Conjugué selon la revendication 1, dans lequel le lipide polaire est sélectionné dans le groupe consistant en sphingosine, céramide, phosphatidylcholine, phosphatidyl glycérol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, cardiolipine et acide phosphatique, sphingomyeline et autres sphingolipides.

3. Conjugué selon la revendication 1 ou 2, dans lequel le peptide est un fragment peptidique de protéines pathogènes et dérivés d'un microbe.

4. Conjugué selon l'une des revendications 1 à 3, dans lequel lors d'une utilisation pharmaceutique le peptide est présenté via le mode de présentation des antigènes du complexe majeur d'histocompatibilité de classe 1.

5. Conjugué selon l'une des revendications 1 à 3, dans lequel lors d'une utilisation pharmaceutique le peptide est présenté via le mode de présentation des antigènes du complexe majeur d'histocompatibilité de classe 2.

6. Conjugué selon l'une des revendications 1 ou 2, dans lequel le peptide est un peptide bloquant d'antigène.

7. Conjugué selon l'une des revendications 1 ou 2, dans lequel le peptide est un peptide bloquant d'antigène du non-soi.

8. Conjugué selon l'une des revendications précédentes, dans lequel le peptide actif antigéniquement comprend 4 à 100 acides aminés incluant les analogues d'acides aminés et leurs dérivés.

9. Conjugué selon l'une des revendications précédentes, dans lequel le peptide est lié de manière covalente via un

résidu d'espacement, ledit espacement comprenant un groupe liant fonctionnel à chaque extrémité.

10. Conjugué selon la revendication 9, dans lequel le groupe liant fonctionnel est faible et l'autre groupe liant fonctionnel est fort.

11. Conjugué selon la revendication 9 et 10, dans lequel l'espacement permet une libération hydrolitique facilitée ou une libération enzymatique facilitée du peptide vers un site intracellulaire.

12. Conjugué selon l'une des revendications 9 à 11, dans lequel le résidu d'espacement est un peptide de formule (acide aminé)$_n$ et n est compris entre 2 et 25 et le peptide est un polymère d'un acide aminé particulier.

13. Composition pharmaceutique comprenant un conjugué tel que défini dans l'une des revendications 1 à 12 et un support pharmaceutiquement acceptable.

14. Vaccin comprenant un conjugué selon l'une des revendications 1 à 12 et un support pharmaceutiquement acceptable.

15. Utilisation d'un conjugué tel que défini dans les revendications 3 à 5 ou 8 pour la préparation d'un vaccin destiné à prévenir des infections par des organismes pathogènes, tels que virus, bactéries, champignons ou protozoaires.

16. Utilisation d'un conjugué tel que défini dans la revendication 6 pour la préparation d'un médicament destiné à prévenir les maladies autoimmunes.

17. Utilisation d'un conjugué tel que défini dans la revendication 7 pour la préparation d'un médicament destiné à prévenir les rejets de transplants de tissus ou d'organes.

18. Utilisation d'un conjugué d'un peptide actif immunologiquement, lié de manière covalente à une molécule transporteuse lipidique polaire et sous une forme autre qu'une forme liposomée, pour la préparation d'une composition pour faciliter l'entrée du peptide dans une organelle sub-cellulaire ou une cellule sélectionnée.

19. Utilisation selon la revendication 18, dans laquelle le conjugué est tel que défini dans l'une des revendications 2 à 12.

FIG. 1

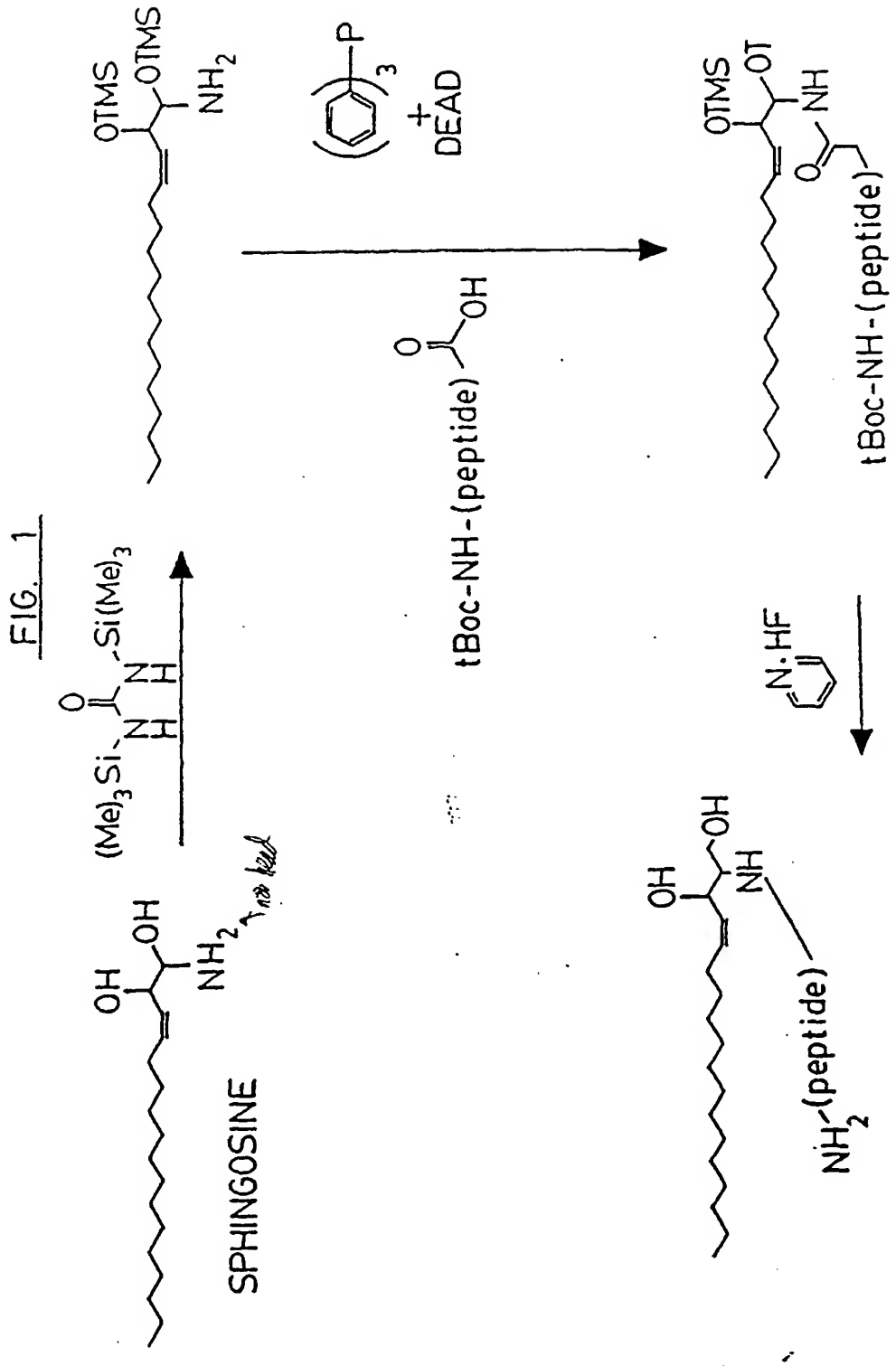

SPHINGOSINE
$(Me)_3Si$
Si(Me)$_3$
OTMS
OTMS
NH_2
P
3
+
DEAD
tBoc-NH-(peptide)
OH
OTMS
OT
NH
tBoc-NH-(peptide)
N·HF
OH
OH
NH
NH_2-(peptide)

FIG. 2

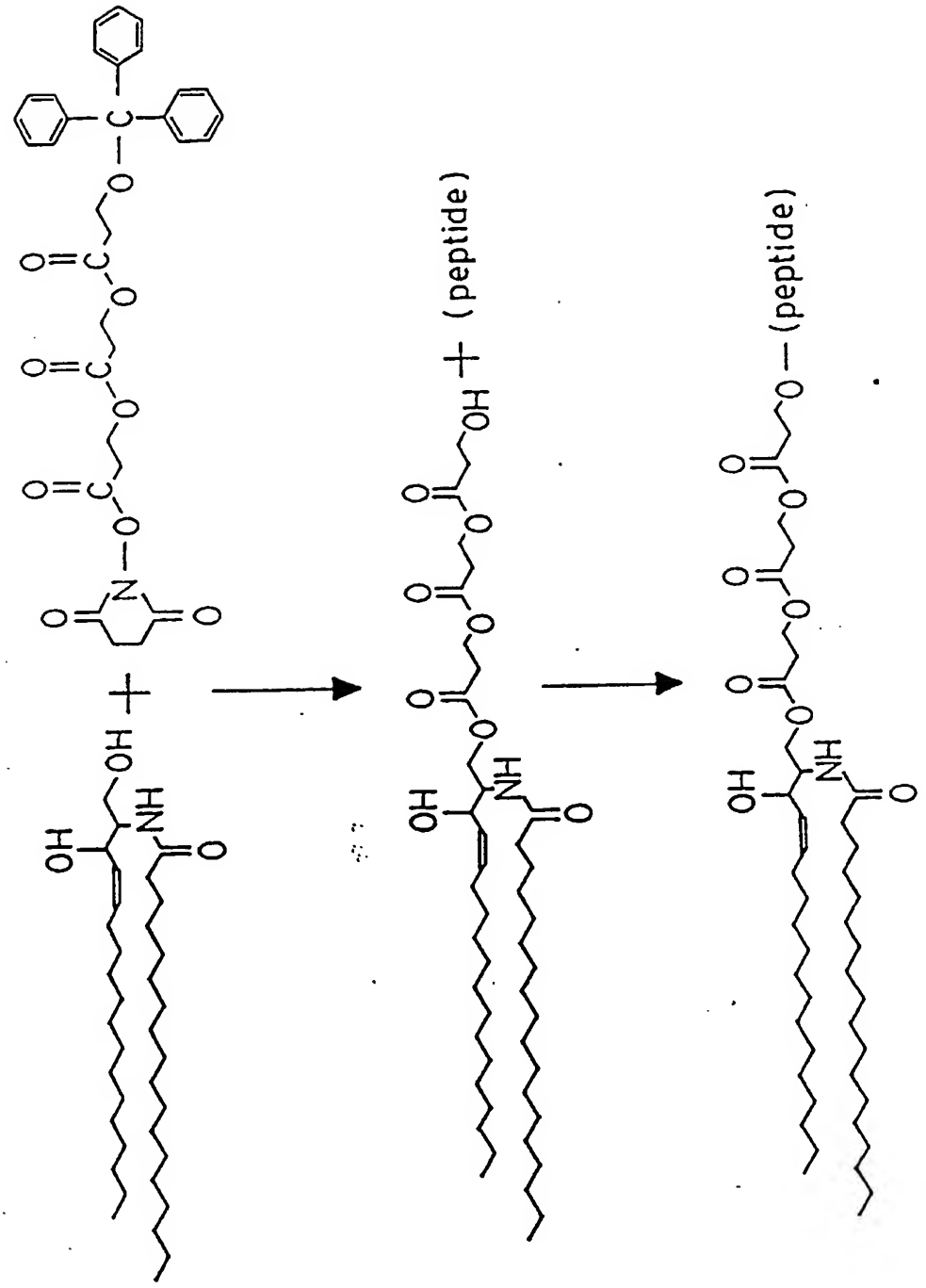


FIG. 3

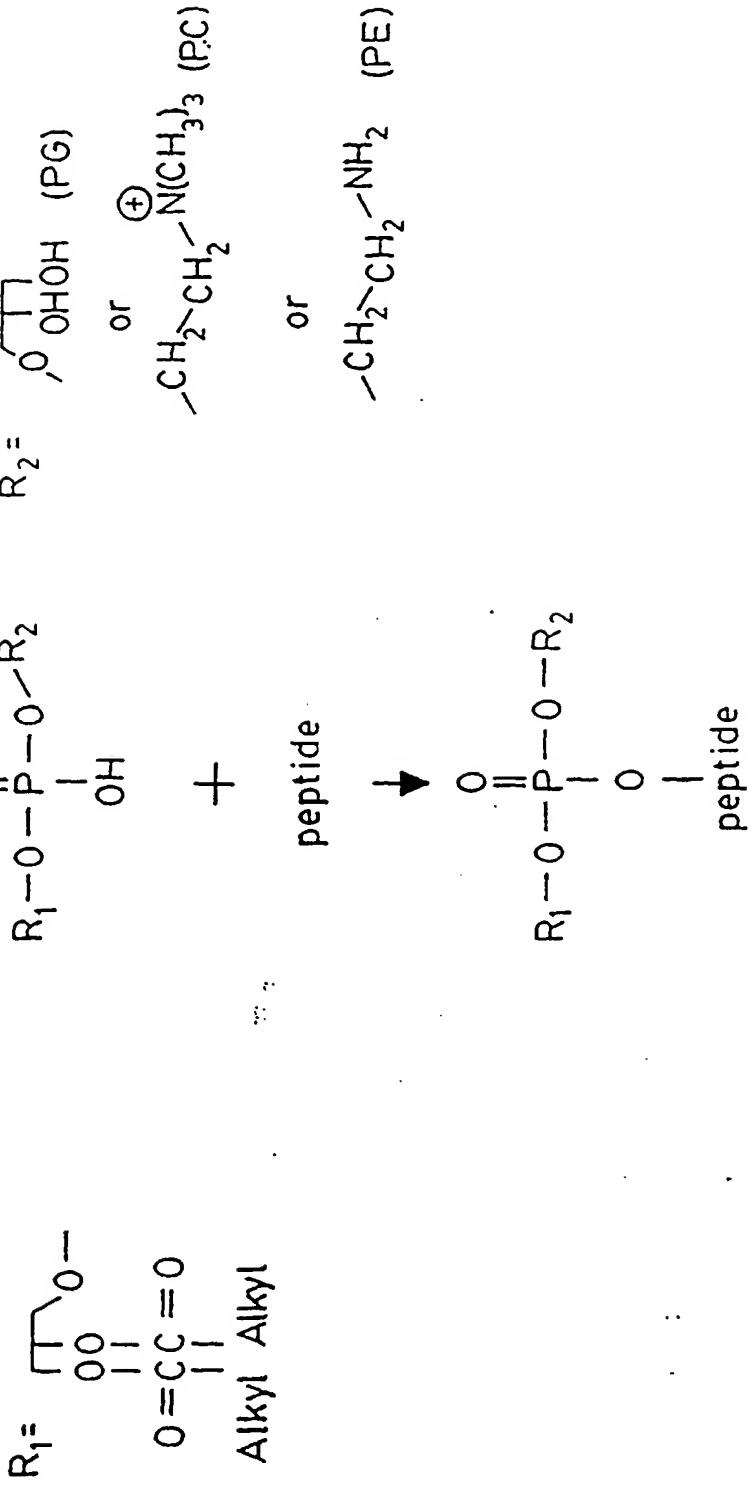

R2=
OHOH (PG)
or
CH2CH2N(CH3)3 (PC)
or
CH2CH2NH2 (PE)
peptide
peptide
R1=
O=C
Alkyl Alkyl

FIG. 4

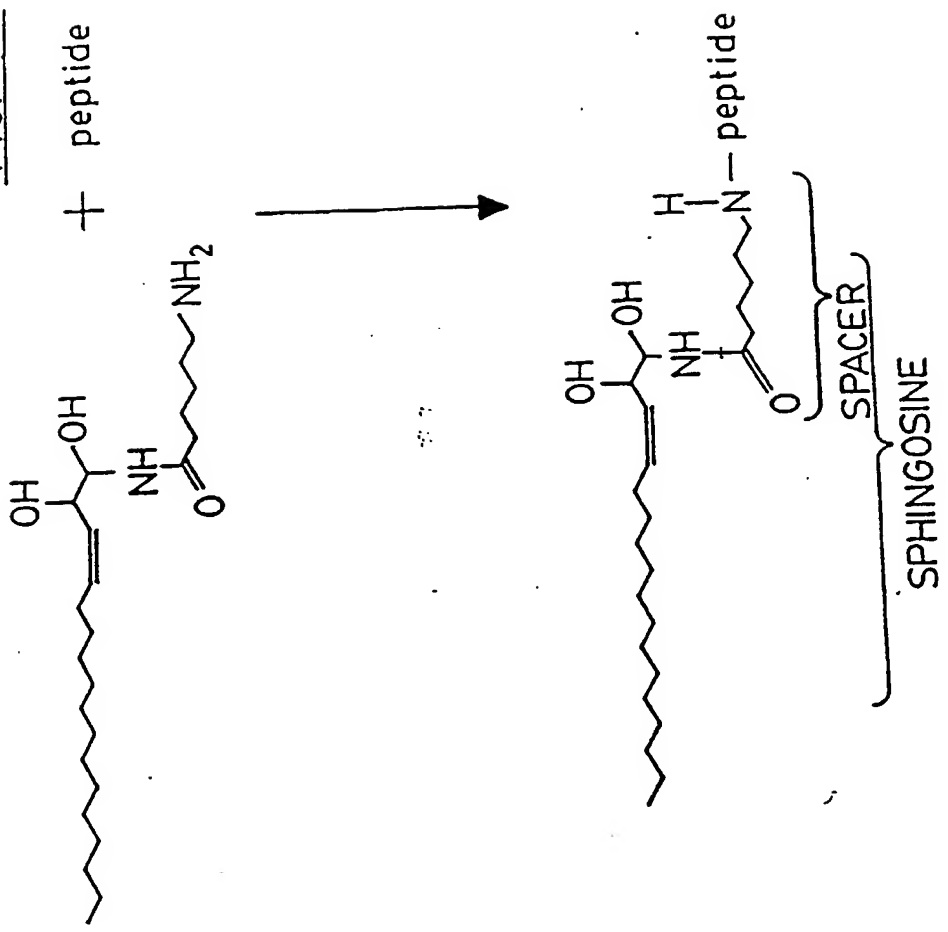

+ peptide
NH2
OH
OH
NH
O
H
N
peptide
OH
OH
NH
O
SPACER
SPHINGOSINE

Fig 5

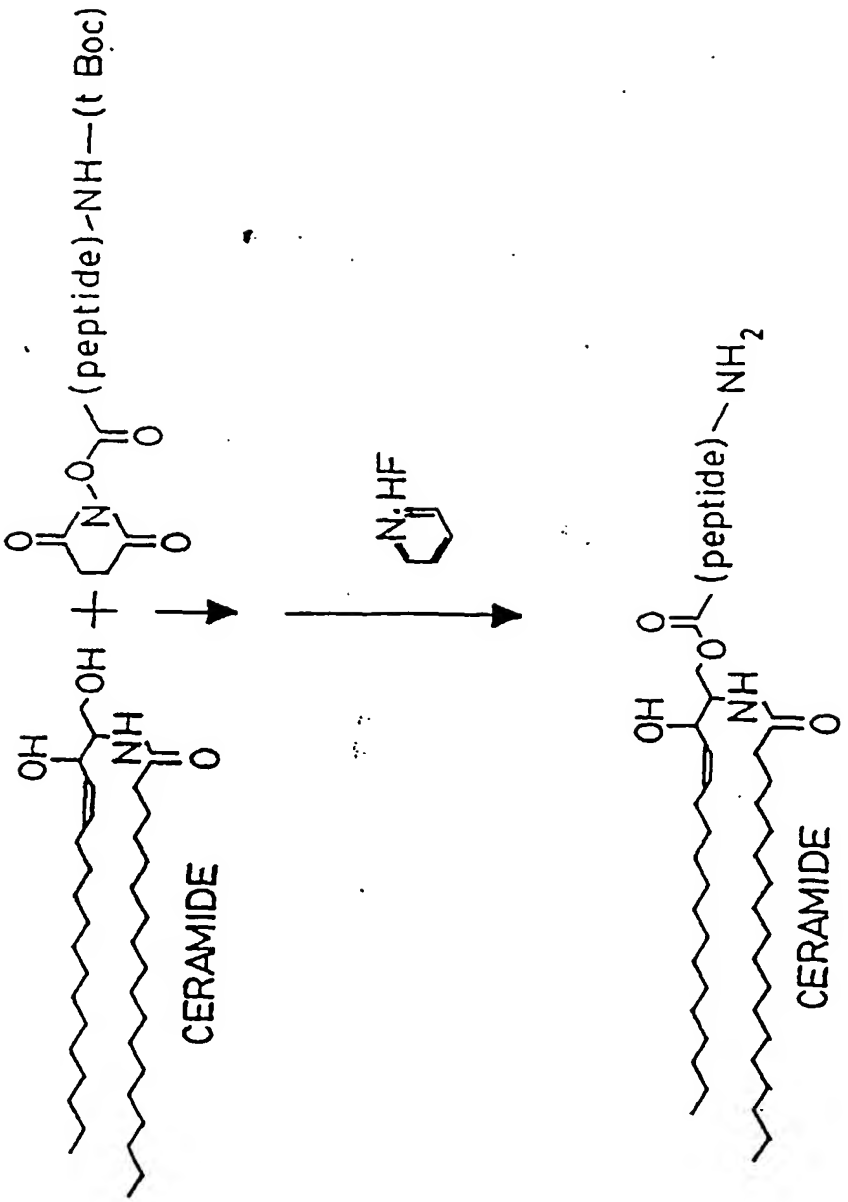


Fig. 6

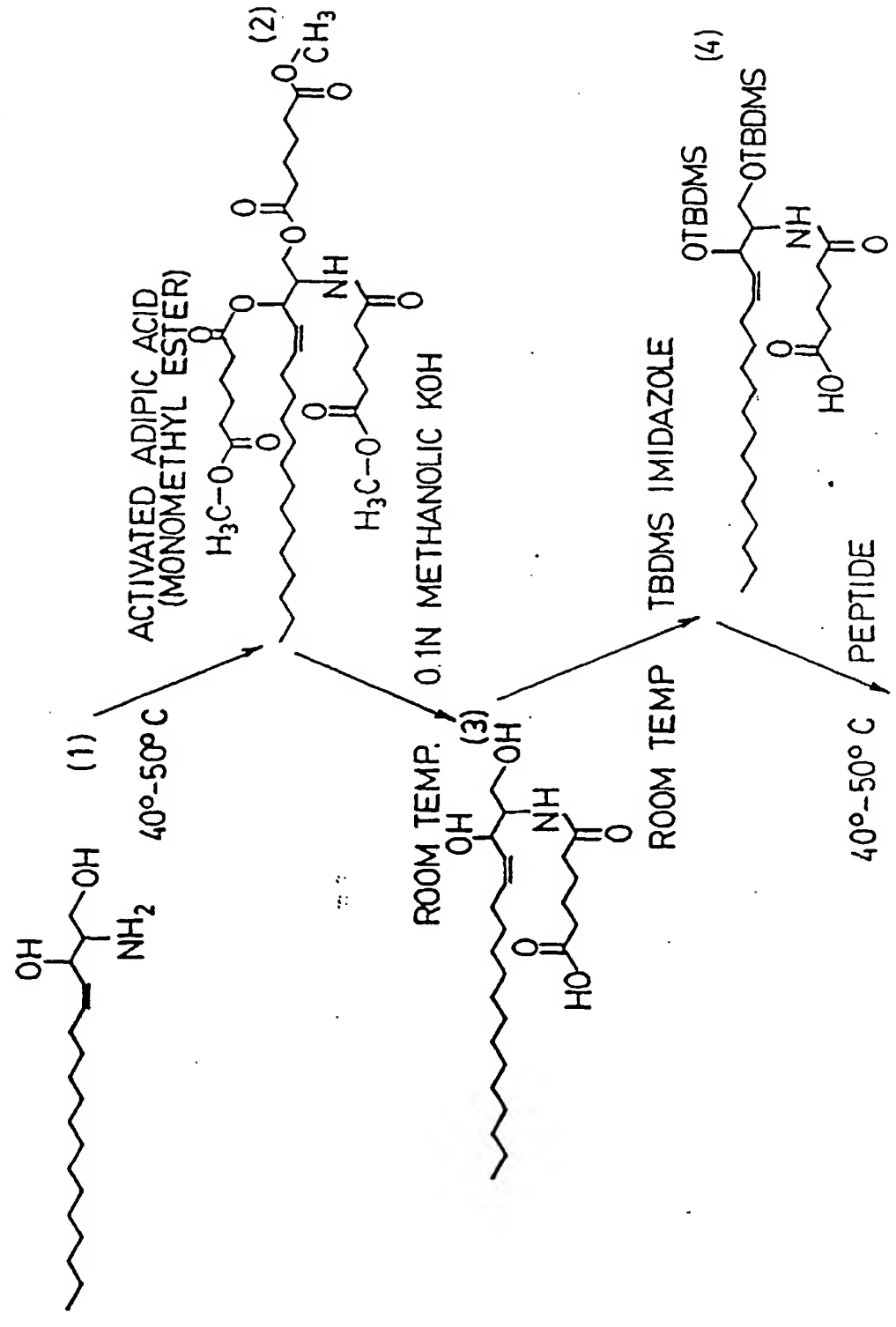

(1)
40°-50° C
ACTIVATED ADIPIC ACID
(MONOMETHYL ESTER)
(2)
ROOM TEMP.
0.1N METHANOLIC KOH
(3)
ROOM TEMP
TBDMS IMIDAZOLE
(4)
40°-50° C
PEPTIDE

Fig. 6 cont'd.

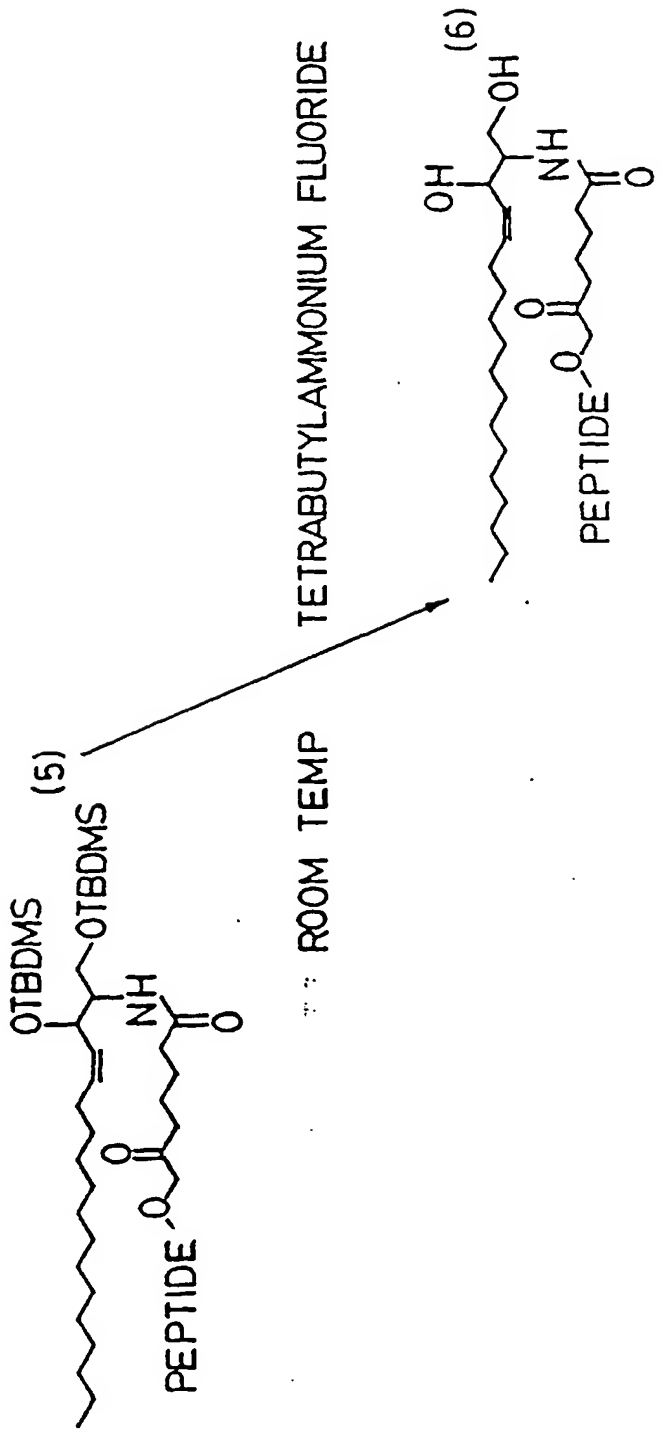
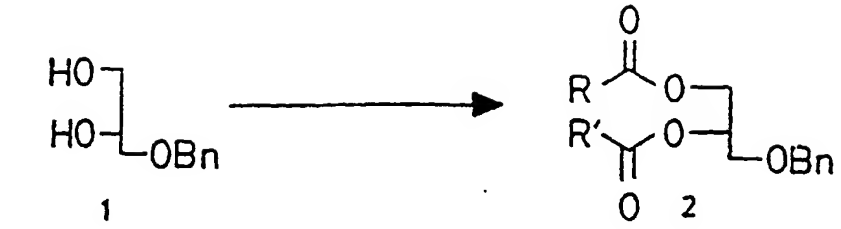
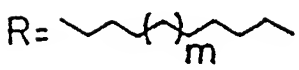
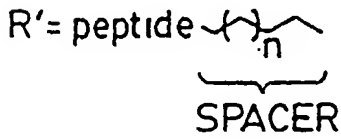
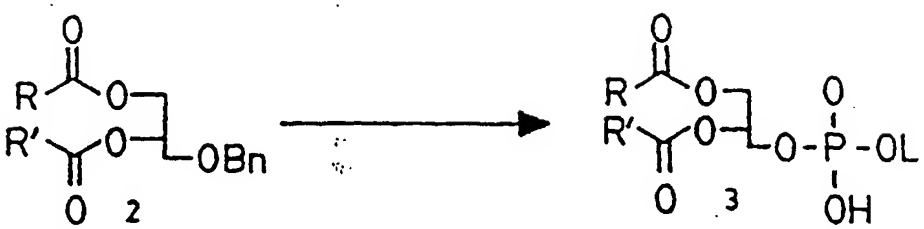

(5)
OTBDMS
OTBDMS
NH
O
O
O
PEPTIDE
ROOM TEMP
TETRABUTYLAMMONIUM FLUORIDE
(6)
OH
OH
NH
O
O
O
PEPTIDE

Fig 7









$$L = \begin{cases} CH_2CH_2\text{-}NH_3 \text{ (PE)} \\ CH_2CH_2\text{-}N(Me)_3 \text{ (PC)} \\ CH(COOH)(NH_3) \text{ (PS)} \end{cases}$$